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14. ABSTRACT
This project takes advantage of a well-characterized mouse model of metastatic breast cancer and use of two photon microscopy on live animal to observe the T cell behavior within the primary tumor and lung metastasis environment after anti-CTLA-4 treatment in presence or not of radiotherapy treatment. We transfected 4T1 tumor cells with a FRET based reporter probe of Caspase 3 activation. It allowed us to follow cell death *in vitro* and *in vivo*. We have also been able to detect cytotoxic T cells expressing Granzyme B within the tumor. These tools can now be used to dissect the cytotoxic activity of TIL after treatment. Interestingly, we have shown that the treatment with anti CTLA-4 antibody 9H10 increases expression of PD-1 on the TIL surface. This finding is of major importance and is consistent with our hypothesis that PD-1 might be implicated in the absence of T cell arrest observed after anti CTLA-4 treatment. We strongly believe that T cell stop is crucial to allow tumor cell elimination. We will then test if treating the tumor bearing mice with anti CTLA-4 and anti PD-1 will allow TIL to stably interact with tumor cell and induce cell death.

15. SUBJECT TERMS
Breast Cancer, Cell migration, CD8 T cells, Cell death. Microscopy

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Note: NYU Medical School has been seriously impacted by Hurricane Sandy in October 2012. Part of the institute is still in process of recovery and experiments have been delayed. The mouse irradiator located on the basement of the building was damaged and is out of order. We are still trying to find the best option to irradiate the mice.

1- Introduction

Blocade of CTLA-4 with antibodies is known to favor anti-tumor immunity. However, anti-tumor activity of anti-CTLA-4 antibodies against poorly immunogenic tumors requires combination with additional treatment as Radiotherapy (RT). The interaction between tumor cells and the immune system is complex and tightly regulated. Visualizing T cells in action give us unique possibility to access information about cell-cell interactions *in vivo* and how it influences the outcome of T cell response. It is likely that in cancer, the cytotoxicity of T cells is influenced by the duration of cell-cell contact. Radiotherapy and anti CTLA-4 treatment as well as PD-1 blocking antibody can modulate the T cell motility and cell-cell interactions. We hypothesize that by supporting stop signal in tumor environment growth of the tumor would be limited. This phenomenon could also play an active role in promoting metastasis elimination.

Our approach aims at characterizing the influence of long lasting interactions induced by NKG2D ligand expression on tumor cell surface and measuring how it influences cell cytotoxicity. We will also assess if decreasing GO signal by blocking PD-1 protein increases the formation of stable interactions with tumor cells.

2- Body

AIM1: Dissecting the rules of NKG2D dependant STOP during the elimination of tumor cells.

Preliminary data from Dr Dustin's and Dr Demaria's labs highlighted the role of NKG2D during RT+anti-CTLA-4 combined treatment (1). During the elimination of a tumor cell, 3 components are of major importance: the effector/target ratio, the duration of effector-target interaction and cell cytotoxicity. We aim at dissecting the influence of NKG2D ligand expression on the surface of tumor cells combined to anti CTLA-4 treatment on these different components.

In order to investigate the role of NKG2D ligand recognition in absence of other signals induced by RT treatment, we started to create vector to transfect the tumor cell line 4T1, with an inductible Tet ON system. Two NKG2D ligands of different affinity will be studied in this part: Rae-1b (KD:345nM) and H60a (KD:30nM) (2-3)

Sequences encoding the genes of interest (here H60a or Rae1b) will be inserted after a promoter containing an improved tetracycline response element (TRE). The second element of the vector is the tetracycline transactivator which is capable of binding to the operator TRE only when bound by doxycycline. As soon as the construct will be available, they will be used to stably transfect the CFP-4T1 cell line by electroporation. The expression of NKG2D ligand will be tested after doxycycline treatment.

One of the goal is to image the lung metastasis of the tumor bearing mice in order to assess the motility cells in this tissue. To do that, CXCR6 $^{GFP/+}$ mice were injected with 4T1 CFP cells, on day 30, metastasis were collected from the lung, maintained in physiological condition of temperature and oxygen and imaged by Two-photon laser microscopy (Figure 1)

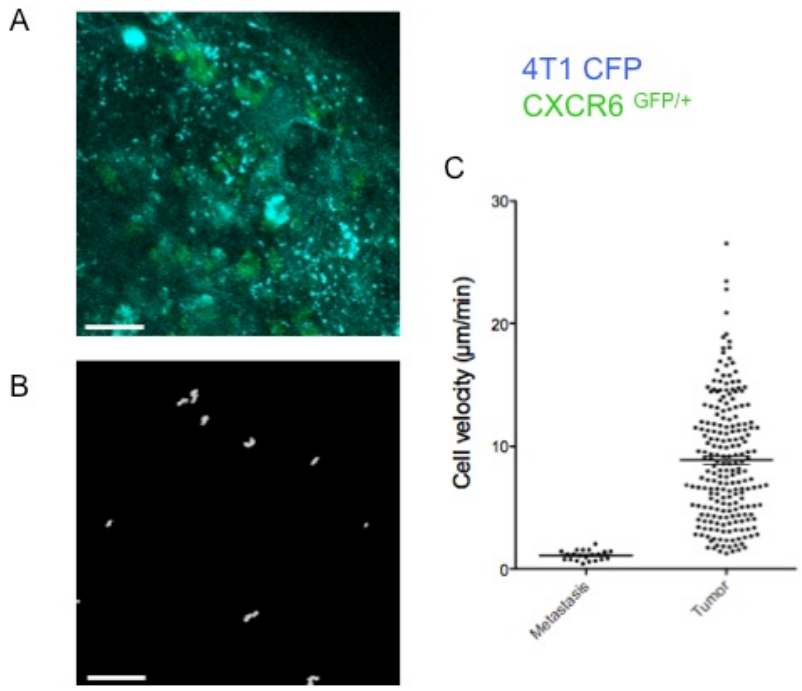

Figure1: Imaging of metastasis

A: Image of a metastase at day 30 after tumor implantation. 4T1-CFP tumor cells (blue), CXCR6 GFP/+ infiltrating cells (green) B: Cell tracking C:Cell velocity in the metastasis compared to tumor site. Scale bar : 20µm

The cell speed was determined using an automated tracking software. As shown in figure 1B-C, the cell velocity is very low in the metastasis compare to tumor. We think that the absence of motility of T cells in the metastasis might be due to hypoxia. To improve the setting, we will try to increase oxygenation of the running medium.

In the tumor, we observed that in the periphery of the tumor, near the blood vessel and the capsule (≤50µm) the cell motility is much higher than it is in the core of the tumor, where no blood vessel are detected (Figure 2). We believe that the absence of motility in the core of the tumor could be due to hypoxia. We will try to inject some anti- MHC antibody in order to determine if the absence of T cell motility in the core is due to antigen recognition or not.

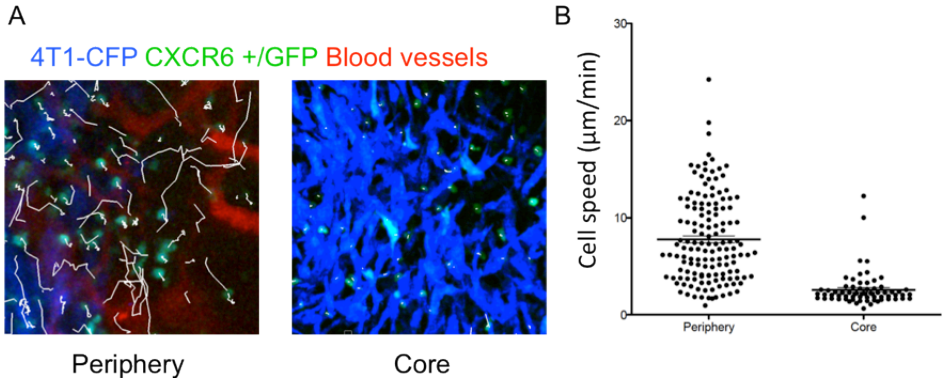

Figure 2 : Motility of CXCR6-GFP+ cells in the periphery and core of the tumor.

A-Image of 4T1-CFP tumor cells (blue), CXCR6 GFP/+ infiltrating cells (green) and blood vessel stained with quantum dots (red). B- Cell velocity in tumor periphery and in tumor core.

AIM2: Measuring Cytotoxicity of TIL after STOP signal induced by combined treatment.

CTLA-4 can specifically inhibit CD8 T cell effector function (4). Antibody blockade of CTLA-4 removes these suppressive signals and allows tumor-specific T-cells (which would otherwise be anergized) to expand and perform effector functions. We propose to study the influence on cytotoxicity of the TIL. The rate at which tumor cells are killed by cytotoxic effectors after RT + aCTLA-4 treatment and a possible role in promoting tumor regression need to be determined.

To quantify cytotoxicity of TIL, granzyme B accumulation was measured on tumor sections. CXCR6$^{GFP/+}$ recipient mice were injected with CFP-4T1 tumor cells. On day 13 and 14, tumors were irradiated and treated with 9H10 antibody (or left untreated for the control). On day 16 tumors were harvested and tissue was fixed and frozen. Tissue section were then stained with anti granzymeB-APC antibody (Figure 3). Granzyme B positive cells were observed in the tumor from mice treated with both IR and 9H10 (Fig 2) at a higher rate than in the control, suggesting that T cell cytotoxicity is induced by the dual treatment. As a future plan, we will try to determine if granzyme B+ cells are located in some specific area of the tissue. We will stain tissue section with blood vessel marker to see if there is any correlation between proximity to blood vessel and granzymeB staining. We also aim at determining if radiotherapy or 9H10 alone can promote cell toxicity and if we can detect GranzB+ and CD107a+ T cell by flow cytometry (in a first experiment we couldn't detect Granzyme B+ cells, data not shown).

These experiment will then be repeated using the TetON-4T1 CFP cells when they will be ready.

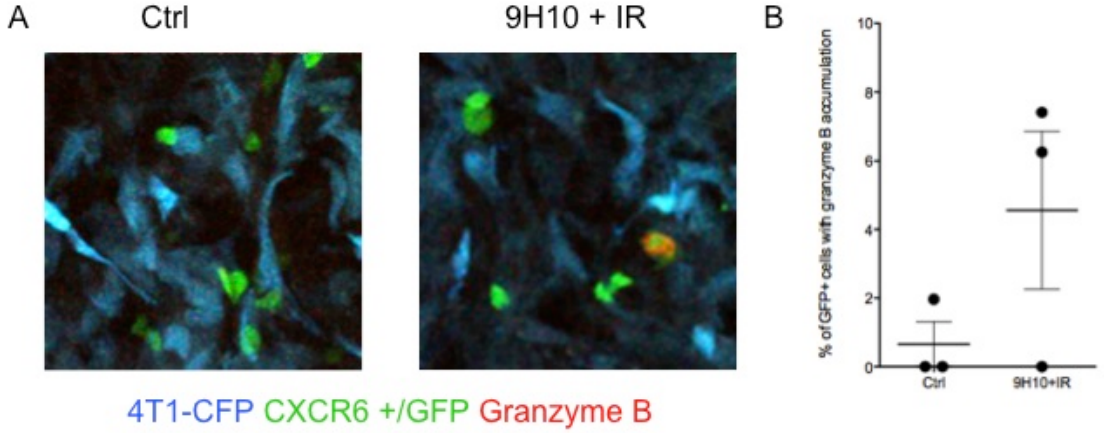

Figure 3: Granzyme B staining on tissue section.
A- Granzyme B staining on tissue section B- Quantification of the percentage of cells showing granzyme B accumulation.

In order to follow cell death in vivo, the 4T1 tumor cells were transfected with the DEVD FRET based construct. CFP and YFP molecules are linked by a peptide containing the caspase 3 cleavage motif DEVD (5). Apoptosis-induced caspase 3 activation resulted in substrate cleavage and FRET disruption. To measure FRET, CFP is excited using a 405 laser (or a Two-photon laser at 800 nm) and the emission of YFP is collected.

First of all, the stability of the transfection was measured. The 4T1-DEVD cells were left in absence or presence (as a control) of the selecting antibody (G418) during 16 days *in vitro*. After 16 days, the fluorescence was measured by flow cytometry, 4T1-CFP cells were used as negative control (Figure 4). The stability of the probe was very good, the fluorescence is maintain as efficiently with or without the antibiotics. The tumor cells can be injected in the mouse and be

image at day 16 without a risk of loosing the probe.

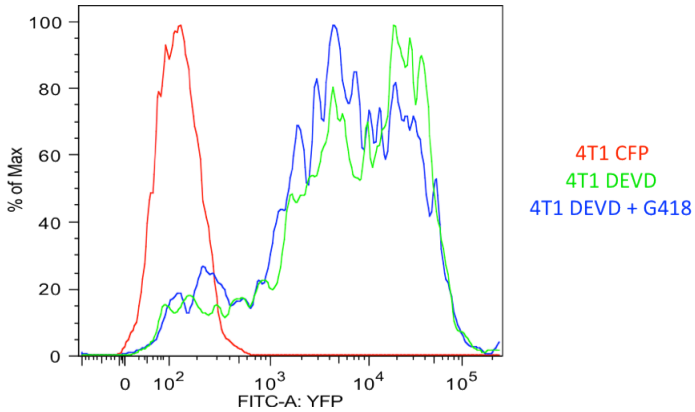

Figure 4 : Stability of DEVD-4T1 reporter at 16 days
YFP fluorescence of tumor cells after 16 days of culture

4T1-DEVD reporter cells were then tested *in vitro*. The cells were exposed to UV and the FRET was measured by flow cytometry. (Figure 5). After 1 hour UV exposure, around 40% of the 4T1-DEVD cells were found to have an increased emission of CFP (disruption of FRET). In order to make sure that this population is ongoing cell death, we will repeat this experiment and stain with a cell death marker such as annexin V.

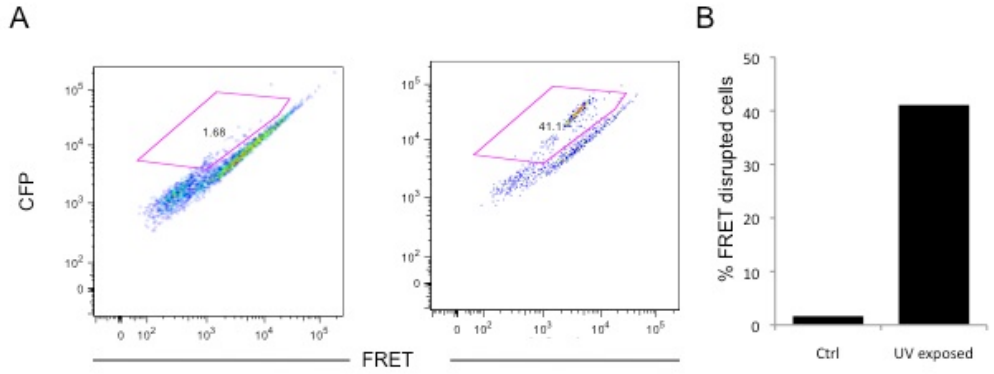

Figure 5: Detecting cell death using DEVD FRET based reporter.
A- FACS plot showing FRET disruption after UV exposure. B- histogram of the % of cells showing a FRET disruption

The 4T1-DEVD tumor cells were then injected in CXCR6 $^{GFP/+}$ mice. On day 13 and 14 mice were treated with radiation (or not as a control) as irradiation is known to strongly increase cell death. Tumors were then observed by 2 photon microscopy to visualize cell death (Figure 6). We observed a very few number of cells showing a FRET disruption (high CFP and low YFP). This number wasn't significantly different between the control and the radiotherapy treated mice (Figure 6B). This could be explain by a very rapid elimination of dead cells. We will repeat this experiment and try to detect cell death in live movies as it was previously done with other tumor models (5).

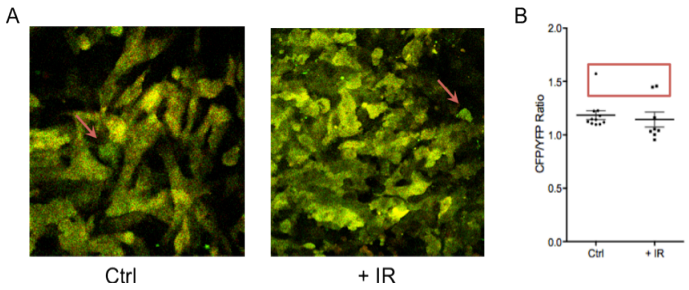

Figure 6: DEVD-4T1 visualisation in tumor tissue in vivo
A-Images of DEVD-4T1 tumors, CFP (green) and YFP (red). FRET disruption increase the CFP emission fluorescence. B- CFP/YFP Ratio for the 2 conditions tested.

AIM3: Influence of PD-1 Signalling on TIL Stop in the tumor

1- PD-1 expression after aCTLA-4 treatment

PD-1 is a major component of T cell tolerance to tumor cells and it was observed that 70 % of TIL from a 4T1 tumor express PD-1 (6). Surprisingly, CTLA-4 blockade induces expression of PD-1 on TIL (7). In order to investigate the potential role of PD-1 in our breast cancer model, Balb/c recipient mice were injected with 4T1 tumor cells. On day 15, mice were treated or not with anti CTLA-4 antibody. PD-1 expression on TIL surface was determined by flow cytometry on day 16 after tumor implantation after anti CTLA-4 treatment (Figure 7)

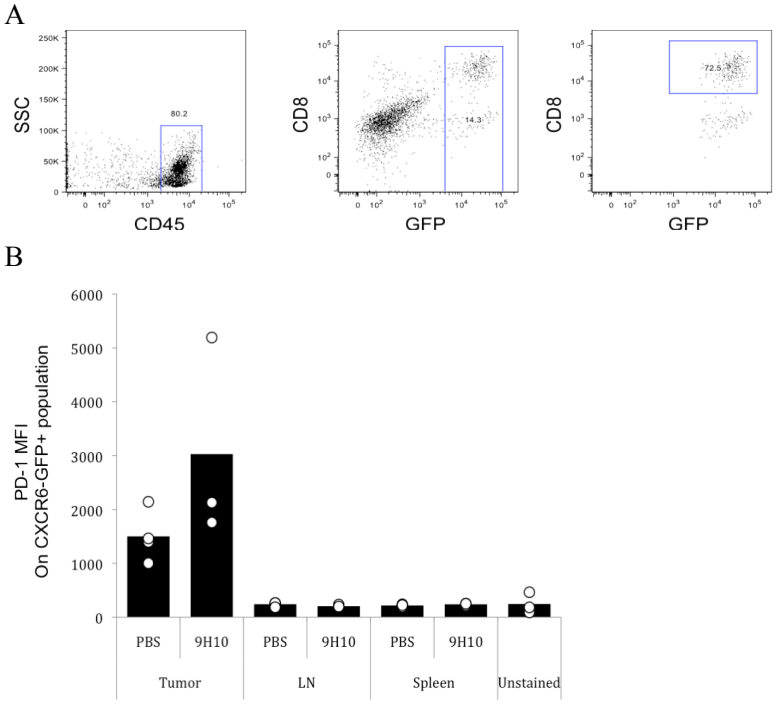

Figure 7: PD-1 expression on GFP+ TIL after 9H10 treatment
A- Flow cytometry plots of TIL in the digested tumor. B- PD-1 Mean Fluorescence intensity in the tumor, Lynph nodes and spleen of tumor bearing mice.

In the tumor, around 80% of the cells recovered after tissue digestion are CD45+. Within this population 15 % are GFP+ (more than 75% of the CD8 population express GFP). Around 70% of the GFP population is CD8 + T cells.

PD-1 expression was determined on the CXCR6 GFP+ population (Mean Fluorescence

intensity is shown on figure 7B). PD-1 expression on the GFP+ TIL in the tumor is higher than in the spleen or lymph nodes. Interestingly, PD-1 expression is also increased by anti CTLA-4 treatment.

2- Measurement of the anti-tumor effect of PD-1

PD-1 was shown to be recruited at the synapse (8) to play an inhibitory role so we can hypothesized that PD-1 plays a role in the absence of stop signal observed after aCTLA-4 single treatment. In order to prevent PD-1 / PDL1 interaction in our model, we will treat the mice with PD-1 blocking antibodies. Tumor growth and lung metastasis will be determine when tumor bearing mice will be untreated, treated with anti-PD-1 antibody alone or in combination with aCTLA-4 antibody.

Even if the anti PD-1 antibody we are using (clone RMP1-14) was described as a blocking antibody (9) we wanted to first make sure that the binding of the anti PD-1 doesn't influence the cell motility. Therefore, we analyzed the transmigration of cells on transwell coated with ICAM1 as well as cell motility on glass coated with ICAM1.

CD8 T cells purified from CXCR6 $^{GFP/+}$ were activated in vitro with antiCD3/CD28 beads. At day 5, cells were collected and used for transwell or glass migration assay. Transwells inserts or glass were coated with ICAM1 (2.5µg/ml). For transwells assays, cells are added to the upper well (in presence or not of anti CD3, 9H10 and anti PD-1) and the percentage of transmigrated cells is counted after 3 hours. For glass assay, cells are added to the wells (in presence or not of anti CD3, 9H10 and anti PD-1), and images are acquired on the microscope. Cell are then tracked using Imaris software to determine cell speed (Figure 8).

As expected, anti-CD3 prevents cell migration in both experiments. We couldn't repeat the increase in velocity previously observed in the lab (1) after 9H10 treatment. As expected, we didn't observe an effect of anti PD-1 treatment, confirming that the biding of the antibody to activated CD8 T cell doesn't modify their motility on ICAM1.

We decided to order a new lot of 9H10 antibody before repeating the experiment as the 9H10 treatment had no effect. Moreover, the cells used here were activated using anti CD3/CD28 beads and non transgenic WT-CD8 T cells, whereas the cells previously used were transgenic CD8 Tcells activated 5 days with peptide. We will also determine if the type of activation will influence this phenomenon.

We can also conclude that the transwell assay and the cell migration assay give very similar results. As transwell allow to test a high number of condition in one experiment, it will be used (prior to migration on glass) if needed.

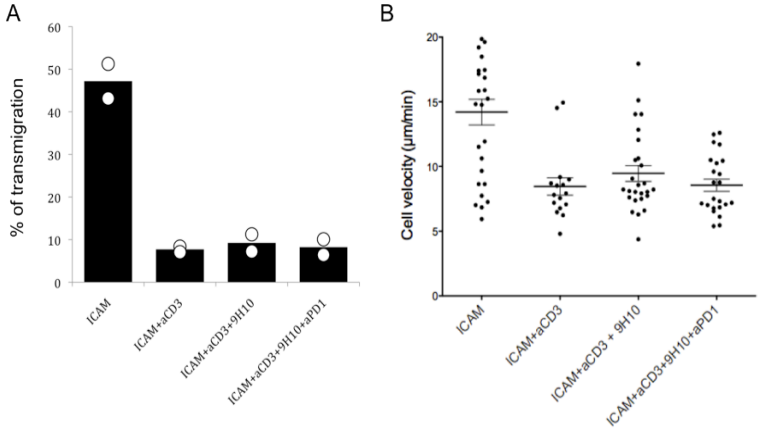

Figure 8 : Activated T cell migration on transwells and glass

A- % of cell transmigration after 3hours on transwell coated with ICAM1 . B- Cell speed on glass coated with ICAM1.

3-Key research accomplishments

- Granzyme B can be detected inside the tumor
- Tumor Core and periphery T cells show different behaviour
- 4T1 cells were stably transfected with DEVD probe
- DEVD-4T1 cells can report cell death in vitro
- PD-1 expression in increased by antiCTLA4 treatment

4-Reportable outcomes

The results obtained during this first year weren't presented yet. But I plan on presenting them during the coming year.

5-Conclusions

Breast cancer remains a therapeutic challenge and understanding how the treatments influence the cells dynamics within the tumor environment is of major influence to optimize current immunotherapeutic strategies. Here we have described some preliminary data on this project that are very promising. We have been able to detect cytotoxic T cell and detect tumor cell death within the tumor *in vivo.* We can now use these tools in order to determine the influence of the anti CTLA-4 and radiotherapy influence T cell cytotoxicity. In another hand, we have shown that anti CTLA-4 treatment increases the expression of PD-1 on the surface of TIL. This observation is consistent with our hypothesis that the absence of stop signal observed in vivo after the anti CTLA-4 treatment might be due to PD-1 upregulation and this question will be addressed soon with anti PD-1 treatment in vivo. We strongly believe that in order to allow cell elimination it is critical to have treatment that are compatible with T cell stop in order to allow tumor cell killing, and that the first results obtain are promising in this direction.

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